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14. ABSTRACT
Prostate cancers that recur after so-called androgen-ablation therapy ("CR-CaP") are typically more aggressive, more likely to spread to local lymph nodes and bones, and less likely to respond to second-tier treatments, and therefore, contribute to significantly decreased patient survival. We posit that enzymes called Src-family kinases (SFK) are required for the progression to CR-CaP, and thus, targeting these enzymes should prevent CR-CaP formation of suppress their growth. We will use animal models of human and mouse CR-CaP in conjunction with genetic and biochemical experiments to show that SFK are critical to the formation of CR-CaP, and thus, are therapeutically targetable using SFK-specific drugs. Our important pre-clinical studies on the critical role played by SFK in CR-CaP disease will serve as the foundation to establish immediate clinical trials in which CaP patients are treated with drugs such as KX2-391 at the commencement of androgen-deprivation therapy.

15. SUBJECT TERMS
Prostate cancer, Src, androgen receptor, castration recurrent, CWR22, TRAMP, tyrosine kinase inhibitors

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Introduction

We are studying the role of Src-family kinases (SFK) in promoting castration-recurrent prostate cancer (CR-CaP) using genetic and pharmacological approaches along with several animal models of CR-CaP. Our synergistic collaboration is based the expertise of the initiating PI (Gelman) in the molecular signaling of SFK in cancer progression, combined with the expertise of the partnering PIs in the CWR22 and TRAMP CR-CaP mouse models (Mohler and Smith, respectively), and in the role of neuroendocrine cells (NE) in the progression of CR-CaP (Smith).

Body

The following is a description of our synergistic research accomplishments in the past year in relation to the specific components of the original SOW (bolded).

***Task 1*. Produce CWR22 cells with tetracycline-regulated Src or Lyn-shRNA expression**

Accomplishments to date-

We have procured and/or produced the necessary Src- and Lyn-specific shRNAs and cloned them into constitutive and inducible lentivirus vectors as described in the grant. These vectors express GFP as a marker of virus infection (driven by an IRES element in the virus construct), and in the tetracycline-inducible system, the lentivirus construct that expresses the tTR tet-inducible transactivator, also expresses a DsRed cassette downstream of an IRES.

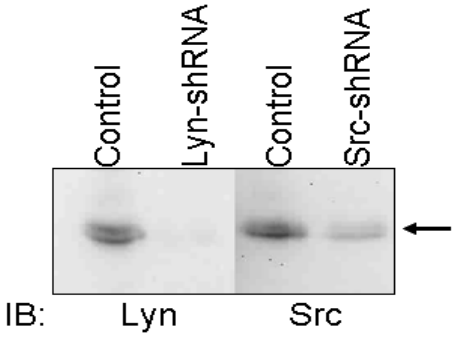

Fig. 1. IB of Lyn and Src in 293T cells infected at an MOI>1 with control, Src- and Lyn-shRNA lentiviruses.

These vectors were tested for their ability to knockdown human Src or Lyn protein levels. Thus, 293T cells were infected at multiplicities >1 GFP-forming virus/cell, and after 3-4 days of culture, the cell lysates were probed for Src or Lyn levels by immunoblotting (IB) with specific monoclonal antibodies (MAb). Fig. 1 shows that both shRNAs were able to knock down their respective targets roughly 8- to 10-fold compared to cells infected with control virus.

We then tested whether these viruses could efficiently infect primary cultures of androgen-dependent CWR22 tumor cells taken from male SCID mice that were implanted with sustained release testosterone pellets. Thus, tumors around 250 mm^3 were removed, converted into single cell suspensions by incubation with collagenase, washed and the cell suspension infected with a titer of control or Src-shRNA lentiviruses that should yield roughly 90% infection. Fig. 2 shows that >90% of the CWR22 cells showed the surrogate GFP marker for virus infection under these conditions, and indeed, this was even higher than the infectivity of 293T cells with the same virus stock.

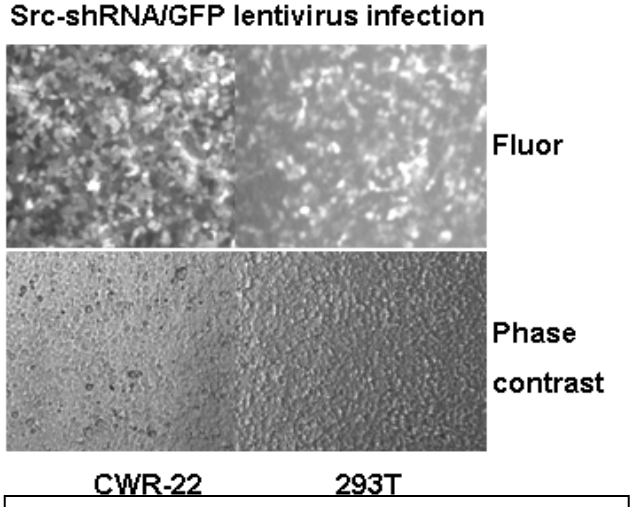

Fig. 2. Fluorescence (Fluor) and phase contrast microscopy of primary CWR22 and 293T cells infected with Src-shRNA lentivirus at an MOI=0.9.

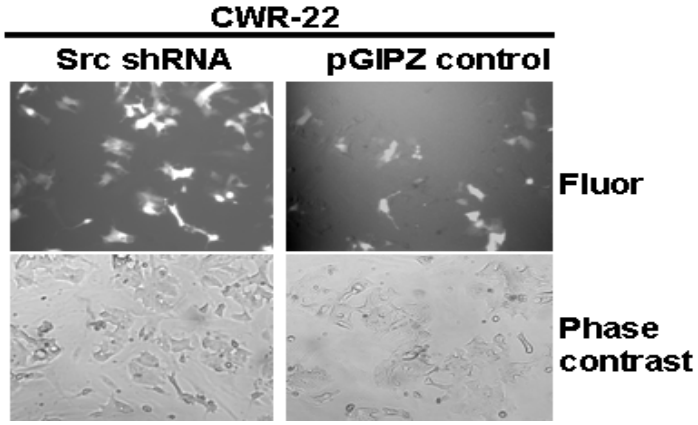

Fig. 3. Fluorescence (Fluor) and phase contrast microscopy of primary CWR22 and 293T cells infected with Src-shRNA or control pGIPZ lentivirus after three passages *in vitro*.

The primary CWR22 tumor cells could be passaged at least three times until they began to senesce, but they retained their GFP expression during this period (roughly 2 weeks) as shown in Fig. 3. The ability to isolate single-cell populations of primary CWR22 cells, to efficiently transduce these cells, and then to reintroduce them into SCID mice is not a trivial accomplishment. This success will allow us to continue our projected studies on the role of SFK and androgen receptor tyrosine phosphorylation in models of CR-CaP.

The synergy in this Task is based on the production and testing of the lentiviruses by the Gelman lab, and the production of the CWR22 primary xenografts by the Mohler/Smith labs though the RPCI Mouse Tumor Model Resource.

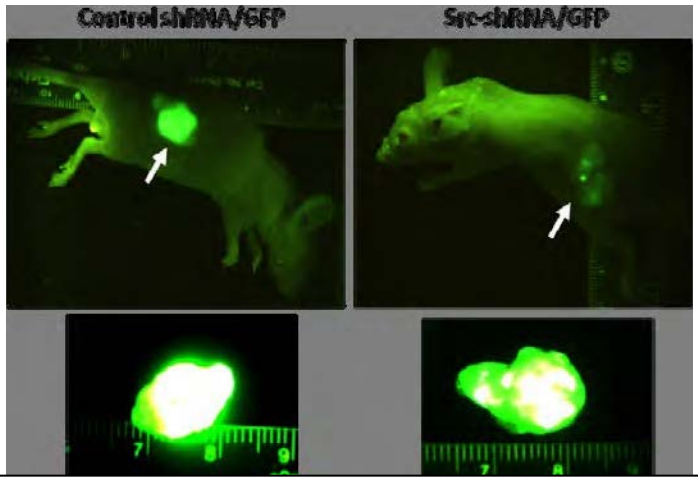

Fig. 4. Recurrent control- and Src-shRNA post-castration CWR22 tumors retain lentivirus GFP expression.

Task 2. **Inject SCID mice with CWR22 Src- or Lyn-shRNA (or vector) cells**

Accomplishments to date - We have infected our first set of primary cultures of CWR22 tumor cells harvested from tumors in testosterone-pelleted male SCID mice (as described in Task 1) with control, Src- and Lyn-shRNA lentiviruses, and these cells have been reinjected s.c. at 10^6 cells + Matrigel into fresh testosterone-pelleted male SCID mice. Mice were castrated when the primary tumor reached 150-250 mm^3, and the primary tumor regression was monitored. There was a small, but statistically significant decrease in the rate of primary tumor regression in the group receiving the Src- vs. the control shRNA. Tumor recurrence has been monitored for roughly 5 months, and to date two control-shRNA tumors have recurred whereas only one Src-shRNA tumor has recurred. These mice will be monitored for another 2-3 months to determine if the control-shRNA groups display higher levels of recurrence. Notably, the recurrent tumors retain their strong lentivirus-encoded GFP expression (Fig. 4), although we will determine whether Src knockdown is maintained in the Src-shRNA tumors.

The synergy in this Task is based on the production of the lentiviruses by the Gelman lab, and the production of the CWR22 primary xenografts by the M ohler/Smith labs though the RPCI Mouse Tumor Model Resource.

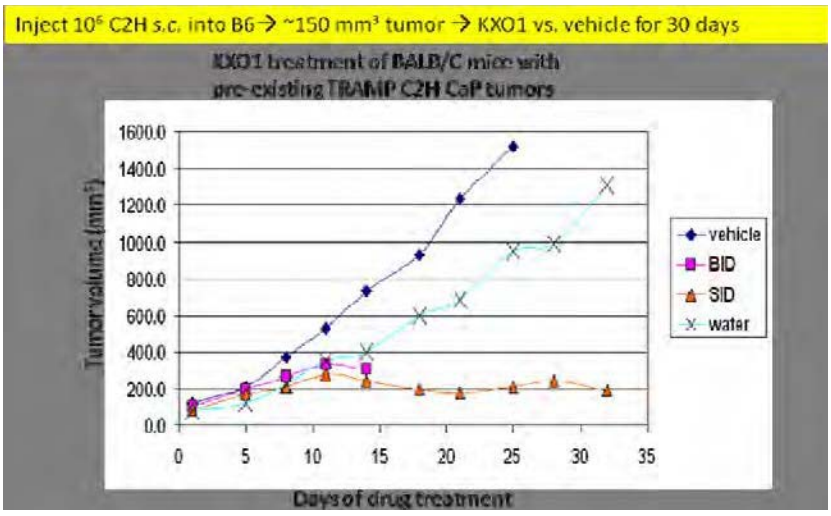

Fig. 5. Dosing of KXO1 as either twice (BID) or once (SID) daily orally, or constant dosing in the drinking water ("water"), vs. vehicle.

Task 3. **Test whether KX2-391 (vs. vehicle or Dasatinib) can prevent recurrent AI-CaP or NE malignancy**

Accomplishments to date- Castrated male C57BL/6 mice injected with TRAMP-C2H (AI-CaP) cells were treated with KX O1, Dasatinib or vehicle. We compared KXO1 dosing protocols and found that except for a lower general toxicity, there was no difference in the suppression of tumor growth by KX O1 dosed once vs. twice daily by oral gavage (Fig. 5). In contrast, constant dosing by adding the drug to the drinking water (assuming 11 ml water intake/20g mouse daily) was less effective at suppressing primary AI-tumor growth. This experiment is currently being repeated with a SID comparison to Dasatinib on primary AI-tumor growth. We started a metastasis experiment with mice whose primary C2H tumors were removed surgically, which were then treated with a 28-day regiment of KXO1, Dasatinib or vehicle

SID. The mice are being monitored for recurrent tumor growth at the primary site and for metastasis to draining lymph nodes. Whereas 6/10 mice receiving vehi cle showed significant primary-site recurrences, only 1/10 mice in the KX O and Dasatinib groups showed recurrence, and these were small lesions. Data are forthcoming on the LN metastases.

T-pelleted, castrated male nude mice have been injected with CWR22, and then their pellets removed and 28-day treatment courses with KX O1, Dasatinib or vehicle. These mice will be monitored for tumor recurrence over the next 4-8 months.

The synergy in this Task is based on the combined efforts by all three PIs' labs in regards to the mouse models and use of the Src-targeting drugs.

Task 4. **Determine if AR^{Y534E} induces recurrent AI-CaP in Src- or Lyn-shRNA CWR22 cells**

Accomplishments to date- Starting with an HA-tagged AR expression vector from Betty Wilson (UNC), we produced an HA-tagged AR^{Y534E} mutant expression vector. This has been verified by sequencing and is now undergoing testing for expression stability in 293T cells.

Task 5. **Demonstrate increased NE proliferation in recurrent AI-CaP (CWR22) or NE (TRAMP) lesions**

Accomplishments to date- Tumors are being isolated and are awaiting staining with appropriate markers.

Task 6. **Produce Src-/- or Lyn-/- TRAMP mice, test for post-castration NE malignancy progression**

Accomplishments to date- We have procured Src-/- mice (C57BL/6) from a pathogen-free facility (SUNY at Buffalo) and Lyn-/- frozen embryos from Jackson Labs. We have started to cross the TRAMP/TRAMP mice into the Src-null background.

Task 7. **Transduce Src- or Lyn-null TRAMP early CaP cells with AR^{Y534E} or WT-AR, test for AI growth in castrated TRAMP mice**

Accomplishments to date- these experiments have not been started yet.

Task 8. **Analyze the role of SFK in NE-mediated AI-CaP growth human AD-CaP cell lines**

Accomplishments to date- these experiments have not been started yet.

Task 9. **Analyze the role of SFK in NE neuropeptide secretion**

Accomplishments to date- these experiments have not been started yet.

Task 10. **Analyze the role of SFK in NE proliferation and neuropeptide secretion *in vitro***

Accomplishments to date- these experiments have not been started yet.

Key Research Accomplishments

-production of Src- and Lyn-shRNA lentiviruses (constitutive and inducible expression)

-successful efficient transduction of primary androgen-dependent CWR22 tumor cells with shRNA-encoding lentiviruses

-demonstration of Src and Lyn knockdown in human cells using the shRNA-encoding lentiviruses

-re-injection of testosterone pelleted SCID mice with transduced primary CWR22 cells

-demonstration of sustained lentivirus expression (based on GFP expression) for >5 months *in vivo* in castration-recurrent CWR tumors.

-demonstration that KXO1 and Dasatinib can suppress AI-CaP recurrence at the primary site in the TRAMP-C2H model.

-demonstration that KXO1 can suppress the growth of primary-site AI-CaP (C2H) and that once daily oral dosing is as potent as twice daily (with lower toxicity).

Reportable Outcomes
None.

Conclusion
The project is progressing at pace with no major obstacles. The synergistic component of the award has been critical to our success thus far and to our projected ability to proceed with the outstanding tasks. This project could not have been accomplished by each of the indivi dual labs. Specifically, this project is progressing strictly because of the combining of the various expertise, such as the active use of the CWR22 and TRAMP models, the isolation and identification of NE cells, and the development and use of the shRNA-encoding lentiviruses. The PIs have also had scheduled meetings to strategize and to review data.

References None

Appendices None